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Thomas Langan 10/96

PI - Signature Date

Introduction:

In the initial year of the grant, we have carried out the bulk of the studies to characterize breast cancer cell lines with respect to overexpression of cyclin D1 protein and the presence of Rb (retinoblastoma) protein (Specific Aim No. 1). In addition, we have begun examination of surgically obtained breast cancer tissue samples for cyclin D1 protein overexpression and Rb protein content (Specific Aim No. 6).

Since the original grant proposal was submitted, advances in our understanding of cell cycle regulation, and in particular advances in our understanding of the importance of defects in cell cycle regulation to the development of cancer, have proceeded at a rapid pace( )( ). In order to incorporate these advances into our investigation of cell cycle regulatory defects in breast cancer, we have expanded and reoriented some aspects of our approach. This has included a detailed assessment of the levels of cyclin dependent kinase (cdk) 4 and cdk 6, and of the cdk4/cdk6-specific cdk inhibitor protein p16 (see **Results** below). This reorientation of the investigation in the light of ongoing research advances can be accommodated within the framework of the originally requested funds and resources.

Materials and Methods:

Breast cancer cell lines and tumor material

A panel of 13 breast cancer cell lines were obtained both from the American Type Culture Collection (BT-483, DU4475, MDA-MB-157, MDA-MB-175VII, MDA-MB-361, MDA-MB-415 and Hs578T), and the University of Colorado Tissue Culture Core Facility (COLO 591, MCF-7, MDA-MB-231, MDA-MB-330, T47-D and ZR75.1). Two normal, immortalized, breast epithelial cell lines, MCF-10A and MCF-12A, and one normal breast epithelium cell line transformed with SV40 T antigen (TAg), HBL-100, were also obtained from the University of Colorado Tissue Culture Core Facility. The cell lines BT-483, COLO 591 and DU4475 were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 18 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine. MCF-10A and MCF-12A were cultured in Hams F12/DME (1:1) supplemented with 10% fetal calf serum, 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 μg/ml insulin. Hs578T was cultured in DMEM media supplemented with 10% fetal calf serum, 0.45% glucose, 0.4% sodium bicarbonate and 2 mM L-glutamine. The remaining cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin.

Three unmatched frozen tumor samples and one matched pair of frozen non-tumor and tumor tissues from breast cancer patients undergoing mastectomy were provided by Dr. Wilbur Franklin, University of Colorado Cancer Center Tissue Procurement Core, University of Colorado Medical School.

Antibodies

The following primary antibodies were obtained from Upstate Biotechnology, Lake Placid, NY: anti-cyclin D1, anti-cdk4, anti-cdk6, and anti-PSTAIRE kinase. Anti-cyclin E and anti-p16 were obtained from Pharmingen, and anti-pRb was a gift from Dr. Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX). The horseradish peroxidase-conjugated secondary antibodies were from Bio-rad.

Protein extractions

Cell lines. Cells were harvested from each of two 175 cm^2 flasks at 50-70% confluence. Following resuspension in PBS approximately 2 X 10^6 cells were removed for fluorescence-activated cell sorter (FACS) analysis by the University of Colorado Cancer Center Flow Cytometry Core to determine cell cycle distribution. The remaining cells were resuspended in 4 X Laemmli sample buffer (Laemmli., 1970), boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Tissue samples. Individual pieces of tumor and non-tumor tissues each weighing approximately 0.2 g, were crushed to a fine powder under liquid nitrogen, lysed in 4 X Laemmli sample buffer and processed as described above for the cell lines.

Western Analysis

Approximately 50 μg of each protein extract were subjected to SDS/PAGE (Laemmli., 1970) and transferred either to nitrocellulose (Schleicher and Schuell) for future probing with the anti-p16 antibody or Immobilon P (Amersham) membranes for 45 minutes at 0.45 A using the Genie Electrophorectic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Results:

A breast cancer panel was assembled consisting of 13 breast cancer cell lines, three non-tumor cell lines, one pair of non-tumor and tumor tissues derived from a breast cancer patient, and three unpaired tumor tissues derived from breast cancer patients. The tumors and cell lines involved in this study represent several different histopathological types and were derived from both primary tumors and metastatic deposits. The ages of the women from whom tumors originated ranged from 23-74 years (Table 1).

Cultures of the cell lines were harvested for protein extraction at 50-70% confluence and their cell cycle distribution assessed by FACs analysis. The percentage of cells in S-phase ranged from approximately 15% to 40%. Approximately 50 μg of protein derived from both the cell lines and tumor samples were subjected to SDS/PAGE, blotted, and probed with 8 anti-cell cycle protein antibodies. The cell lines were initially immunoblotted with the anti-Human cdc2 Kinase (PSTAIRE), the latter of which appears to be expressed at relatively constant levels in the cell lines. The same pattern of expression was demonstrated following immunoblotting with an antibody specific for one of the PSTAIRE sequence-containing proteins, Cdk2 (Fig.2 and Table 3).

Cyclin D1 and Rb protein expression in breast cancer cell lines and tumors.

Following immunoblotting with anti-Cyclin D1 and anti-Rb antibodies the cell lines and tumors were classified into one of four groups: those that (1) showed the amplification of Cyclin D1 protein in the presence of functional pRb (ie., the hypophosphorylated, active form of pRb); (2) showed the absence/very low level of Cyclin D1 in the absence of functional pRb; (3) showed absence/very low level of Cyclin D1 in the presence of functional pRb; and (4) showed Cyclin D1 protein amplification in the absence of functional pRb. Of the 17 breast cancer cell lines and tumor tissues analyzed, 14 show a correlation between the levels of Cyclin D1 protein and the presence or absence of functional Rb protein. Eight of the breast tumors/cell lines show overexpression of cyclin D1 protein in the presence of functional pRb (classified into group 1). Six of the breast tumors/cell lines show the absence/low level of cyclin D1 protein in the absence of functional pRb (classified into group 2). The results are shown in Table 2 and Fig. 1. Furthermore, the control cell line, HBL-100, that expresses the SV40 Tag (and thus lacks functional pRb), also exhibits very low levels of Cyclin D1 protein. This is consistent with the model that abnormally elevated levels of Cyclin D1 protein are required to overcome the active pRb-mediated G1/S block in breast cancer cells. The correlation between Cyclin D1 overexpression and the presence of functional Rb protein has previously been demonstrated both in non-small cell lung cancer (Schauer et al., 1994) and esophageal cancer (Jiang et al., 1993). Three of the tumor cell lines, COLO 591, ZR75.1 and MDA-MD-175VII displayed an inverse correlation between the levels of pRb and Cyclin D1 protein (group 4) indicating that these cell lines employed different mechanisms to escape the normal cell growth controls. None of the cell lines nor tumor tissues were classified into group 3.

Expression of Cyclin D1-associated Cdk4 and Cdk6 proteins in breast cancer cell lines and tumors.

The levels of the Cyclin D1-associated Cdk4 and Cdk6 proteins remain relatively constant during the normal cell cycle with oscillations in Kinase activity controlled by Cyclin D1 (Hunter and Pines., 1994). Characterization of these proteins in the breast cancer cell lines involved the use of the specific Cdk4 and Cdk6 immunizing peptides to block non-specific antibody binding. Three of the eight tumor cell lines, MCF-7, MDA-MB-231 and ZR75.1, and the non-tumor cell line, HBL-100 (SV40 Tag-transformed) cell line exhibit elevated levels of Cdk4 protein relative to the other tumor cell lines and non-tumor control cell lines. By contrast, the levels of Cdk6 protein do not appear to be significantly increased in the majority of breast cancer cell lines nor tumors tissues relative to the non-tumor controls (Table 3 and Fig. 2). Furthermore two of the breast

cancer cell lines and one of the non-SCLC control cell lines lack detectable Cdk6 protein. This suggests differences in the potentially oncogenic activities of Cdk4 and Cdk6 during tumorigenesis in different breast tumors. Previous studies have indicated possible oncogenic roles for Cdk4 and Cdk6 in tumorigenesis. Tam et al. (1994) described the overexpression of the Cdk6 protein in some types of tumor, and amplification of the both the Cdk4 gene (Khabtib et al., 1993) and the Cdk4 protein (Tam et al., 1994) has been demonstrated in several tumors including breast cancer. Furthermore, a mutated Cdk4 protein has been identified in two primary melanoma tumors that prevents binding of the Cdk-inhibitor, p16, resulting in constitutively active Cyclin D1/Cdk4 complexes (Wolfel et al., 1995). The above data suggest that Cdk4 has the potential to contribute to tumorigenesis through at least two pathways: aberrant overexpression or mutation, resulting in the inhibition of p16-mediated cell cycle regulation.

Expression of the Cdk4-inhibitor p16 is restricted to breast cancer cell lines lacking functional pRb.

The expression of p16 is restricted to two of the cell lines that lack functional pRb and exhibit elevated levels of Cdk4 protein:- HBL-100 and MDA-MB-231 (Table 3 and Fig. 2). The breast cancer cell line, MCF-7, exhibits elevated levels of Cdk4 protein but contains functional pRb (and has been shown to contain an active Cyclin D1/Cdk4 complex by Tam et al., (1994)) and thus does not exhibit detectable levels of p16. These findings are consistent with the model that active pRb inhibits p16 expression and that the loss of Rb protein allows the p16 protein to bind to Cdk4 resulting in the dissociation and inactivation of the Cyclin D1/Cdk4 complex. It is possible that the elevated levels of Cdk4 in MDA-MB-231 may contribute towards overriding the growth regulatory function of p16. It is also possible that MDA-MB-231 contains a mutant Cdk4 protein that prevents binding of p16. Three of the breast tumor tissue samples and the non-tumor tissue control also show varying levels of p16 expression. However, both the non-tumor control and the tumor sample 92 1908 (T) contain low levels of functional pRb. This result suggests that low level expression of pRb may not suffice to completely inhibit p16 expression in breast tissues.

Amplification of Cyclin E protein occurs in a minority of breast cancer cell lines and tumors.

The expression of Cyclin E protein appears to be maintained at a relatively high level in both the breast cancer cell lines and the non-tumor cell lines, MCF-12A and HBL-100 (Table 3 and Fig. 2). In addition, the breast cancer cell line, DU4475 appears to show unusually high levels of the protein relative to the rest of the panel. Since DU4475 lacks functional pRb and thus expresses only a low level of Cyclin D1 protein, it is possible that Cyclin E may play an potentially oncogenic role in late G1 to ensure maintenance of the transformed state. Two of the four tumors also show elevated levels of Cyclin E relative to the normal tissue control and other tumor samples. Like DU4475, one of these tumors, S92 4153 (T), exhibits low levels of Cyclin D1 protein owing to the lack of functional pRb, whereas the other tumor, 92 1908 (T), contains functional pRb and exhibits significant over-expression of Cyclin D1. The latter case suggests that one mechanism of oncogenic transformation in breast tumors involves the sequential phosphorylation (and thus inactivation) of pRb by Cyclins D1 and E (in complex with their catalytic subunits) in mid and late G1 phase respectively, resulting in the inappropriate transition into S-phase. Since the levels of the Cdk2 protein remain relatively constant in the breast cancer cell lines and non-tumor cell lines, it is likely that any potential role in tumorigenesis is achieved by modulation of their catalytic activity by Cyclin E as opposed to changes in the absolute level of protein.

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Table 1. Breast cancer cell lines and tumors.

Cell line/ Tissue	Histology	Sample	Treatment	Age of patient (yrs)
Tumor cell lines				
BT-483	Ductal ca (iv)	Tumor	Unspecified	23
COLO 591	-	Ascites fluid	Unspecified	43
DU4475	Carcinoma	Met cutan. nodule	Chemotherapy	70
MCF-7	Adenoca.	Pleural eff.	Rad/Hormonal	69
MDA-MB-231	Adenoca.	Pleural eff.	Chemotherapy	51
MDA-MB-330	Carcinoma	Pleural eff.	Radiation	43
T47-D	Ductal ca.	Pleural eff.	Unspecified	54
ZR75.1	Carcinoma	Unspecified	Unspecified	-
MDA-MB-157	Medulla ca.	Pleural eff.	Rad/Chemo	44
MDA-MB-175VII	Ductal ca.	Pleural eff.	Unspecified	56
MDA-MB-361	Adenoca.	Brain metastasis	Unspecified	40
MDA-MB-415	Adenoca.	Pleural eff.	Surg/Rad[1]	38
Hs578T	Ductal ca.	Tumor	Radiation	74
Non-tumor cell lines				
HBL-100	Epithelial cells	Milk of nursing mother	None	27
MCF-10A	Immortalized, non-transformed, epithelial cells		None	-
MCF-12A			None	-
Tissues				
S95 2462 (N)]	Normal epithelial	Surgical margin	Surgery	
S95 2462 (T)]	Ductal adenca(iv)	Tumor	Surgery	52
S92 4153 (T)	Ductal ca (iv)	Tumor	Surgery	35
S93 1493 (T)	Ductal ca (iv)	Tumor	Surgery	68
92 1908 (T)	Ductal ca (iv)	Tumor	Surgery	68

[1]this patient did not receive radiation prior to surgery.
Tumor and non-tumor tissues are designated by the patient's number followed by (T) or (N) respectively. The table contains the following abbreviations :- ca, carcinoma; (iv), invasive; Adeno, Adenocarcinoma; Pleural eff., Pleural effusion; Rad, Radiation; Chemo, Chemotherapy; and Surg, Surgery.

Table 2. Cyclin D1 and Rb protein profiles in breast cancer cell lines and tumors.

Cell line/ Tissue name	pRb status	Cyclin D1 protein	Group
MCF-7	+	++++	1
MDA-MB-330	+	+++	1
T47-D	+	++	1
MDA-MB-157	+	+++	1
MDA-MB-361	+	+++	1
MDA-MB-415	+	++++	1
Hs578T	+	+++	1
92 1908 (T)	+	++++	1
BT-483	-	+/-	2
DU4475	-	+	2
MDA-MB-231	-	+/-	2
S95 2462 (T)	-	+/-	2
S92 4153 (T)	-	+/-	2
S93 1493 (T)	-	+/-	2
COLO 591	-	++	4
ZR75.1	-	++++	4
MDA-MB-175VII	-	+++	4
MCF-10A (Non-tumor)	+	+++	1
MCF-12A (Non-tumor)	+	+++	1
S95 2462 (N)	+*	+/-	1
HBL-100 (Non-tumor)	-	+/-	2

Functional pRb (hypophosphorylated form) is indicated by a (+) and non-functional pRb (hyperphosphorylated form) by a (-). *Non-tumor tissue sample exhibits a very low level of functional pRb. The levels of Cyclin D1 protein are indicated as follows:- (++++), very high level; (+++), high level; (++), moderate level; (+), low level; (+/-), barely detectable level; (-), undetectable level. The tumor tissue samples are designated with a "T" or "N" suffix respectively.

Table 3. Expression of cell cycle regulatory proteins in breast cancer cell lines and tumors

Cell line/ Tissue name	Cyclin D1	pRb	p16	Cyclin E	CDK4	CDK6	CDK2
Tumor c. line							
BT-483	+/-	-	-	+	+/-	+/-	+/-
COLO 591	++	-	-	++	+/-	++	++
DU4475	+	-	-	++++	+/-	+++	++
MCF-7	++++	+	-	+++	++	++	++
MDA-MB-231	+/-	-	+++	+++	++	+++	++
MDA-MB-330	+++	+	-	+++	+	+++	++
T47D	++	+	-	++	+	-	++
ZR75.1	++++	-	-	++	+++	-	++
MDA-MB-157	+++	+	nt	++++	nt	nt	++
MDA-MB-175VII	+++	-	nt	+++	nt	nt	++
MDA-MB-361	+++	+	nt	++	nt	nt	++
MDA-MB-415	++++	+	nt	+++	nt	nt	++
Hs578T	+++	+	nt	++	nt	nt	+/-
Non-tumor c. line							
HBL-100	+/-	-	+++	++	+++	++	++
MCF-10A	+++	+	-	nt	+/-	+++	nt
MCF-12A	+++	+	-	+++	+/-	+++	++
Tissue							
S95 2462 (N)	+/-	+*	++	+/-	nt	++	nt
S95 2462 (T)	+	-	++	+/-	nt	++	nt
S92 4153 (T)	+	-	+++	+++	nt	++	nt
S93 1493 (T)	+/-	-	nt	+/-	nt	++	nt
92 1908 (T)	++++	+	+++	+++	nt	++	nt

The levels of particular cell cycle proteins are indicated by (+) and (-) signs as follows:- (++++), very high level; (+++), high level; (++), moderate level; (+), low level; (+/-), barely detectable level; and (-), undectable level; and (nt), not tested.
Functional pRb is designated by a (+) and non-functional pRb by a (-). *Non-tumor S95 2462 (N) expresses a very low level of functional pRb.
Tumor and non-tumor tissue samples are designated by the patient's code followed by a (T) or (N) respectively.

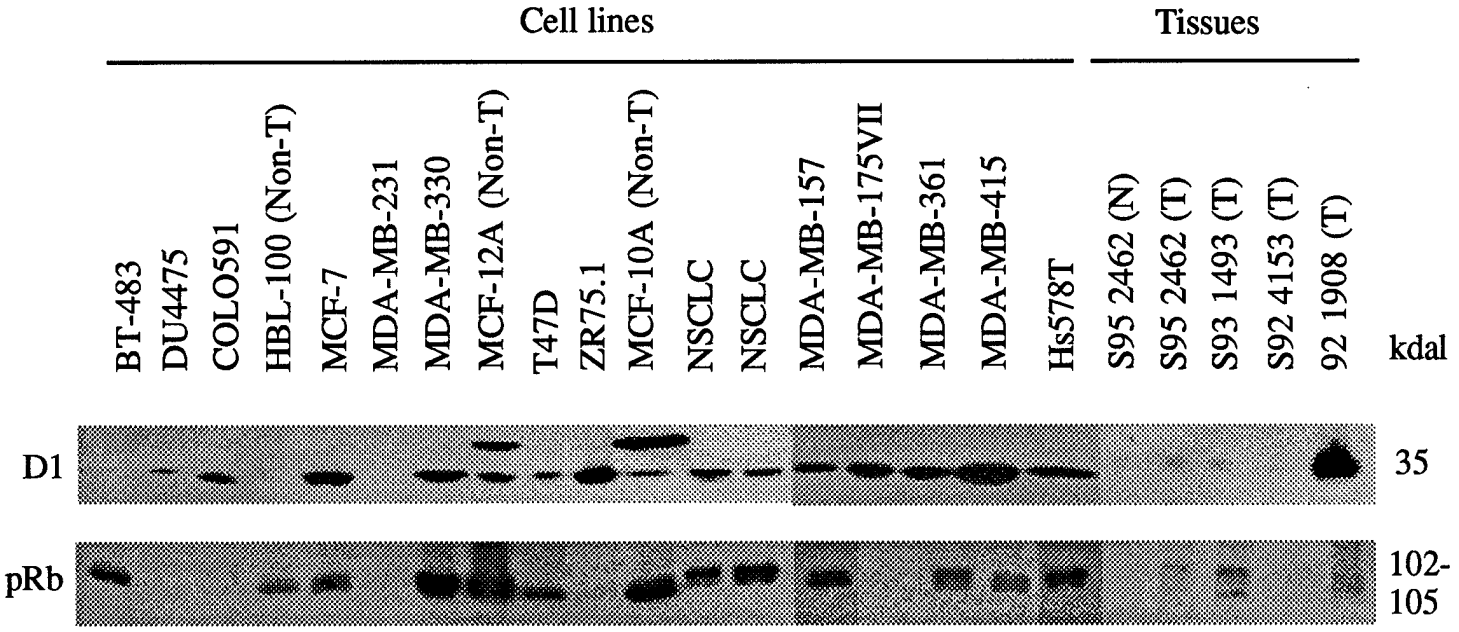

Fig. 1. Cyclin D1 and Rb protein expression in breast cancer cell lines and tumors. The patterns of cyclin D1 and Rb protein expression were determined in a panel of breast cancer cell lines and tumors by immunoblotting. Protein extracts were prepared from the indicated cell lines and breast cancer patient tissues, subjected to SDS/PAGE, transferred to Immobilon-P membranes, and probed with the anti-cyclin D1 and anti-Rb antibodies respectively. The molecular weights of the two proteins are indicated to the right of each panel. Non-tumor control cell lines included HBL-100, MCF-10A and MCF-12A. Two non-small cell lung cancer (NSCLC) cell lines were included for comparison of cyclin D1 and Rb protein expression patterns between tumor cell lines of different tissue origin. Tumor and non-tumor tissue samples are designated with the patient's number followed by a "(T)" or "(N)" suffix respectively.

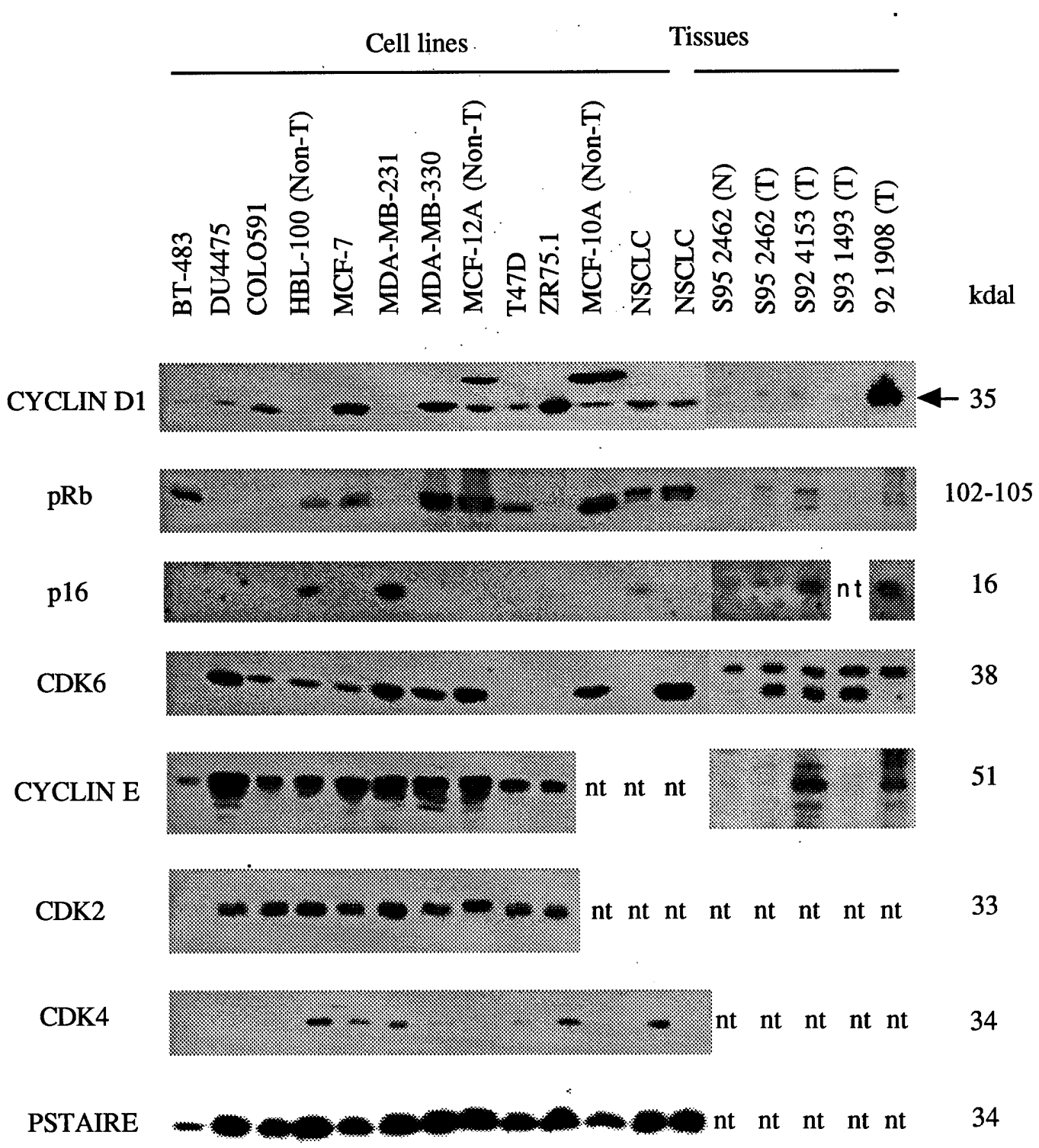

Fig. 2. Expression of cell cycle regulatory proteins in breast cancer cell lines and tumors.

The patterns of expression of cell cycle regulatory proteins were determined in a panel of breast cancer cell lines and tumors by immunoblotting. Protein extracts were prepared from the indicated cell lines and breast cancer patient tissues, subjected to SDS/PAGE, transferred to Immobilon-P membranes (or to nitrocellulose for p16 analysis), and probed with the antibodies indicated to the left of each panel. The molecular weights of the cell cycle proteins are indicated to the right of each panel. Non-tumor, control cell lines included HBL-100, MCF-10A and MCF-12A. To enable a comparison of the expression of cell cycle regulatory proteins between tumor cell lines of different tissue origin, two non-small cell lung cancer (NSCLC) cell lines were included in the panel. Tumor and non-tumor tissue samples are designated by the patient's number followed by a "(T)" or "(N)" suffix respectively. "nt" indicates that the sample was "not tested" for protein expression. The cell lines MDA-MB-157, MDA-MB-175VII, MDA-MB-361, MDA-MB-415 and Hs578T are not included in this figure since their expression analysis is limited to just four regulatory proteins.

Goals for Year 2:

We intend to extend the basic studies on cyclin D1, Rb, cdk4, cdk6, and p16 inhibitor protein as well as, for comparison, cyclin E and cdk2, to additional breast cancer cell lines as well as a larger number of breast cancer tissue samples. These data will be examined for confirmation of the distribution of cell cycle regulatory defects in breast cancer outlined in **Results** above. In addition, we plan to examine the growth factor/serum requirements for cyclin D1 expression (Specific Aim No. 2) and to characterize the complexes formed by cyclin D1 with specific cdks (Specific Aim No. 6). In the light of recent research advances, complexes with cdk4 and cdk6 will be of special interest here. We also will continually reorient our currently planned approaches to reflect other research advances as the work proceeds.